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# EFFECT OF CALCIUM IONS ON ECDYSTEROID SECRETION BY TESTES OF *HELIOTHIS VIRESCENS*

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**Abstract**—1. Testes of *Heliothis virescens* synthesized ecdysteroid in media containing low titers of calcium; the optimum calcium titer for testis sheaths stimulated to synthesize ecdysteroid *in vivo* was *ca* 1 mM, while the optimum for testes stimulated *in vitro* with the peptide testis ecdysiotropin was *ca* 0.3 mM calcium.

2. Verapamil at concentrations lower than $10^{-3}$ M induced increases in ecdysteroid synthesis, indicating more efficient synthesis when calcium influx was inhibited.

3. Hemolymph of *H. virescens* was 7 mM in calcium, while whole testes were maintained at 1–2 $\mu$M calcium.

## INTRODUCTION

Calcium ions are involved in vertebrate processes as diverse as regulation of muscle contraction, secretion of hormones, digestive enzymes and neurotransmitters, transport of salt and water across the gut, and glycogen metabolism in the liver (Rasmussen, 1983). Although not as much evidence has been accumulated for insect systems, similar functions for $Ca^{2+}$ have been indicated (Berridge and Lipke, 1979). Exogenous $Ca^{2+}$ are required for response to eclosion hormone by *Manduca sexta* nerve cord (Truman, 1980) and stimulation of the metabolism of lipid and carbohydrate by fat body exposed to adipokinetic hormone and trehalagon (Steele, 1980). Stimulation of ovarian muscle with the neuropeptide neurotransmitter, proctolin, requires influx of calcium into the oviduts of *Locusta migratoria* (Lange *et al.*, 1987). Controlled release of diuretic hormone in *Rhodnius prolixus* is mediated by activation and inactivation of calcium channels (Maddrell, 1980). Prothoracic glands of *Manduca sexta*, cultured with the neuropeptide, prothoracicotropic hormone (PTTH), secrete increasing quantities of ecdysone and 3-dehydroecdysone as the external calcium concentration is raised, and as the $Ca^{2+}$ flux is increased by calcium ionophore A23187, although basal production is not affected (Smith and Gilbert, 1986). However, Dale and Tobe (1988a,b) reported little effect of calcium-free media or calcium channel blockers on release and synthesis of juvenile hormone (JH) by corpora allata from *Locusta migratoria*. Nevertheless, $Ca^{2+}$ flux induced by the ionophore, A23187, increased hormone synthesis and release.

Testes of lepidoptera synthesize several ecdysteroid analogues *in vitro* (Loeb *et al.*, 1982; Shimizu *et al.*, 1985), although 20-hydroxyecdysone (20HE) is the primary ecdysteroid analogue secreted by testes of *Heliothis virescens* (Loeb and Woods, 1989), *Lymantria dispar* (Loeb *et al.*, 1988) and *Ostrinia nubilalis* (Gelman *et al.*, 1989). Fused testes from late last instars of *H. virescens*, previously activated *in vivo*, require no additional stimulus to produce ecdysteroid *in vitro* (Loeb *et al.*, 1982; Shimizu *et al.*, 1985; Gelman *et al.*, 1989). However, paired testes from early last instar *H. virescens* can be induced to synthesize ecdysteroid *de novo* when incubated with brain extracts containing the peptide, testis ecdysiotropin (TE) (Loeb *et al.*, 1987, 1988). In this work, synthesis of immunodetectable ecdysteroids by both types of testes from *H. virescens* was found to be optimal in low titers of exogenous $Ca^{2+}$. The nature of this dependence was explored by subjecting both types of testes to a range of $Ca^{2+}$ titers, verapamil, a calcium influx antagonist (Rosenberger and Triggle, 1978), and calcium ionophore A23187, which induces calcium transport across membranes (Pressman, 1976).

## MATERIALS AND METHODS

### *Experimental animals and organs*

*H. virescens* were obtained as pupae from the Cotton Insects Laboratory, Phoenix, AZ, and allowed to emerge as adults. Eggs were collected and the resulting larvae were reared and staged as described (Loeb and Hayes, 1980). Testes were dissected from *H. virescens* at mid-last larval instar (digging stage, days 3 and 4) when they synthesize detectable quantities of ecdysteroid only in the presence of TE and after testis fusion (buried stage, day 5), just prior to pupation, when testes secrete ecdysteroid *in vitro* without addition of TE (Loeb *et al.*, 1984, 1987).

### *Incubation media*

Standard Ringer was 173 mM in sodium, 1.8 mM in calcium, 3.7 mM in potassium, 180 mM in chloride and 2.8 mM in bicarbonate. Calcium-free Ringer was prepared by substituting sucrose (Sigma) (3.6 mM) to maintain osmotic properties. Analysis by flame absorption spectroscopy indicated the actual $Ca^{2+}$ concentration in calcium-free Ringer was $4.6 \pm 0.88$ $\mu$M. However, calcium-free Ringer plus $10^{-4}$ M EDTA was rendered 0.01 $\mu$M in free calcium according to computer aided calculations (Fabiato, 1988). Ringer containing other $Ca^{2+}$ titers (0.1 and 0.5 mM) was prepared with sucrose-substituted stock to maintain the osmotic properties of the original Ringer solution. Verapamil HCl (Sigma) was prepared as a $10^{-2}$ M solution in calcium-free Ringer with 1% DMSO (Sigma, St. Louis,

MO) to aid solubility; subsequent dilutions were made in 0.5 mM $Ca^{2+}$ Ringer. A solution of 50 mM in calcium ionophore A23187 (Sigma) was prepared in 0.5 mM $Ca^{2+}$-Ringer, with DMSO (6%) to induce solubility. In experiments using verapamil and A23187, DMSO was added to control incubate wells at the highest DMSO concentration used in the experimental wells. DMSO at 1% or less did not affect ecdysteroid secretion by testis sheaths (unpublished data). Since *H. virescens* testes are stimulated to synthesize greater amounts of 20HE *in vitro* in the presence of small amounts of 20HE (Loeb *et al.*, 1986), 20HE (Calbiochem Corp, San Diego, CA) at a concentration of 5 pg/$\mu$l was incorporated into all incubation media. Gentomycin sulphate (Sigma) (1%) was also included to retard bacterial growth. EDTA (Sigma) ($10^{-4}$ M) was used as $Ca^{2+}$-free media for 0 $Ca^{2+}$ controls in calcium dose-response experiments.

*Testis ecdysiotropin*

Brains of day 4–6 pupae of *H. virescens* or day 6–7 pupae of *Lymantria dispar* were stored separately at −20°C in methanol:acetic acid:water:thiodiglycol, 90:9:0.9:0.1 (by vol). Brains were sonicated in this solution. The supernatants obtained after centrifugation (1470 *g*) were used after drying an appropriate amount without heat (Speed Vac concentrator, Savant, Farmingdale, NY) and reconstituting it in incubation medium just prior to use. Since TE originating from either species induced synthetic activity in testes of either species (Loeb *et al.*, 1988), TE-active brain extract from either species was used as generic material. Brain extracts were prepared from batches of only 10–30 brains at this stage of the work; at least five different lots of brain extract were used, with equivalent results. The number of brain equivalents used per well (0.5–1) corresponded to the maximum TE activity for that preparation, as indicated by its dose response.

*Radioimmunoassay (RIA) for ecdysteroid*

Enough methanol to produce a 67% solution was mixed into each sample after incubation. Samples were sonicated (Heat Systems) and centrifuged at 1470 *g*; resulting supernatants were dried and subjected to RIA as described by Borst and O'Connor (1974). Ecdysteroid antibody A1 had been prepared by W. E. Bollenbacher against a hemisuccinate derivative of ecdysone coupled to thyroglobulin at the C-22 hydroxyl group (Gilbert *et al.*, 1977). [$^3$H]-Ecdysone (63.5 Ci/mmol) (New England Nuclear Corp., Boston, MA) served as the ecdysteroid competitor; 20HE was used to construct the standard curve for each analysis.

*Calcium determinations*

Testes were rinsed for a few sec in each of three washes of calcium-free Ringer to remove adherent hemolymph, and placed in calcium-free water for subsequent treatment. Samples were prepared for analysis by dry ashing at 480°C in a muffle furnace overnight, and later diluted to appropriate volumes with 5% nitric acid and 8-hydroxyquinoline (4% by weight). Samples were analyzed by flame atomic absorption spectrometry (air–acetylene flame) at 422 nm against standards prepared in 8-hydroxyquinoline. A reference material (Diet RM8431a) with known $Ca^{2+}$ content was analyzed with the unknown samples to ensure the accuracy of the analyses.

*Calculation of organ volumes*

The lengths and widths of 10 representative testes were measured with the aid of an ocular micrometer in an eyepiece of a dissecting microscope; volumes were calculated as in Loeb *et al.* (1984), using the formula for the volume of a prolate spheroid $V = 4/3\pi ab^2$, where $a$ is the radius of the long dimension and $b$ is the radius of the short dimension. Volumes of testis sheaths were estimated by subtracting the mean volume of a hypothetical uniform testicular lumen from the mean volume of the whole testis. The radii of the lumena were determined by subtracting half the width of representative testis sheaths (suspended in Ringer solution) from mean radii of whole testes. Mean volumes of last instar stage D3–D4 and last instar stage B5 *H. virescens* testes were 1.25 mm$^3$ and 4.25 mm$^3$, respectively (Loeb *et al.*, 1984).

*Experimental design*

Freshly dissected testes were washed well in Ringer and soaked for 0.5 to 1 hr in the Ringer with the lowest concentration of $Ca^{2+}$ used in the current series of experiments. TE-stimulated testes were incubated whole in 200 $\mu$l of solution in wells of sterile 24-well plates (Falcon), in randomly chosen groups of two testes per well. Since fused testes were larger, and ecdysteroid production occurs primarily in the sheath of the testis (Loeb *et al.*, 1982), it was possible to use tissue from the same fused testis in several situations. Each organ was cut with a surgeon's scalpel as carefully as possible into eight equivalent segments (estimated by eye) and rinsed in appropriate Ringer to remove testicular fluid and spermatocysts. Fragments were distributed into each of eight wells of a sterile 24-well plate. Six to eight pieces of sheath, derived from as many testes, were incubated per well of a set. This method served to counter some of the variability between testes and at the same time, provided a set of eight wells for comparison purposes. Approximately the same amount of ecdysteroid was produced per testis whether it was incubated whole or cut for use as a sheath preparation (Loeb *et al.*, 1982). In each experiment, two or four wells were reserved for controls wherein testes, or testis fragments were incubated in original Ringer (for comparison to substituted Ringer solutions) or calcium-free Ringer containing $10^{-4}$ M EDTA (for $Ca^{2+}$ dose response experiments) or Ringer containing 0.5 mM $Ca^{2+}$ (for A23187 and verapamil dose response experiments). All experimental and control samples were duplicated in each experiment; data for each experiment is presented as the mean of the duplicates. For each well containing testes or testis fragments, a replicate well containing solutions but no tissue was incubated; after RIA, the amount of ecdysteroid detected in the replicate was subtracted from the amount in the well containing tissue. Tissues or organs were incubated for 3 hr on a rotating table at room temperature. Incubation was stopped by the addition of 400 $\mu$l of methanol to each well. Wells were sealed with strips of parafilm and stored at −20°C until processed for ecdysteroid RIA.

*Presentation of data*

Calcium dose response information is presented as observed. However, the response of each preparation was somewhat different, so that typical data from one experiment are shown here. Other data are normalized by expressing them as the ratio of the mean quantity of ecdysteroid produced in treated (experimental) wells divided by the mean quantity of ecdysteroid produced in control wells.

## RESULTS

### *Effects of* $Ca^{2+}$ *concentration on RIA-detectable ecdysteroid synthesis*

Typical responses of fused *H. virescens* testis sheaths (solid line, circles) and non-fused testes stimulated to secrete ecdysteroid with TE (dotted line, triangles) to a range of calcium concentrations are shown in Fig. 1. The curves are each representative of only one experiment since the data varied somewhat in each run. However, the mean optimum $Ca^{2+}$ titer for induction of immunodetectable ecdysteroid synthesis by fused testis sheath fragments was

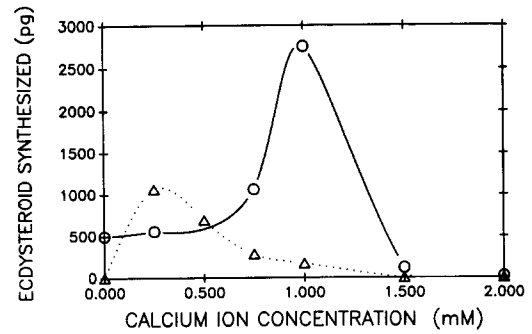


Fig. 1. Typical effects of $Ca^{2+}$ concentration on immunologically detectable ecdysteroid synthesis by testis sheaths from fused testes of *H. virescens* previously stimulated *in vivo* (solid line), and *H. virescens* testes stimulated to secrete ecdysteroid with TE (dotted line). Each point is the mean of duplicates. Fused testis sheath fragments ($n = 4$ experiments) produced maximal immunoreactive ecdysteroid at $0.94 \pm 0.16$ mM $Ca^{2+}$; non-fused testes stimulated with 0.5–1 brain equivalents of TE produced maximal immunodetectable ecdysteroid synthesis at $0.37 \pm 0.11$ mM $Ca^{2+}$ ($n = 5$ experiments). Differences between the $Ca^{2+}$ optima for fused testis sheath secretion and TE-induced secretion were statisticaly different at the 0.995 percentile (Student's *t*-test).

$0.94 \pm 0.16$ mM $Ca^{2+}$ ($n = 4$ experiments); in contrast, younger testes stimulated with 0.5–1 brain equivalent of TE produced maximal immunoreactive ecdysteroid in media containing $0.37 \pm 0.11$ mM $Ca^{2+}$ ($n = 5$ experiments). Some synthetic activity was often detected when external calcium concentrations approached zero. The differences between the $Ca^{2+}$ optima for TE-induced secretion and fused testis sheath secretion were statistically different at the 0.995 percentile (Student's *t*-test).

*Verapamil*

In all cases, ecdysteroid secretion was inhibited at concentrations above $10^{-3}$ M verapamil (Fig. 2). Ecdysteroid synthesis in sheath preparations of *H. virescens* incubated without TE was stimulated approximately six-fold in $10^{-4}$ M verapamil (Fig. 2, solid line). However, verapamil from $10^{-4}$ to $10^{-11}$ M

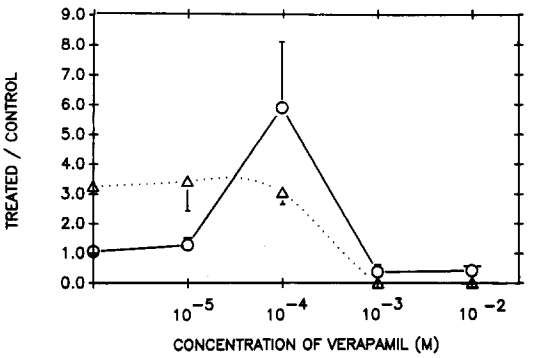


Fig. 2. Effects of verapamil on synthesis of immunologically detectable ecdysteroid in *H. virescens* fused testis sheath tissue (solid lines), and non-fused testes stimulated with TE (dotted lines). The Y-axis represents synthesis in verapamil/synthesis in control (0.5 mM $Ca^{2+}$) Ringer. $n = 6$–8 at each data point. Bars indicate the SEs of the means.

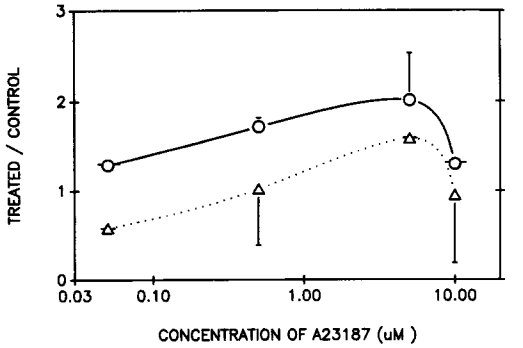


Fig. 3. Effect of calcium ionophore A23187 on synthesis of immunologically detectable ecdysteroid in *H. virescens* fused sheath preparations (solid lines) and *H. virescens* non-fused testes stimulated with brain extracts containing TE (dotted lines). Bars indicate SEs of the means.

(Fig. 2, dotted line) was stimulatory to ecdysteroid synthesis by younger testes incubated with TE. Dilution to $10^{-12}$ M returned TE-stimulated secretion to that of controls (not shown in Fig. 2).

*Effects of calcium ionophore A23187*

Calcium ionophore A23187 was slightly stimulatory to ecdysteroid secretion by fused testes at concentrations from 0.05 to 5 $\mu$M, but had no significant effect at the highest concentration tested, (10 $\mu$M) (Fig. 3, solid line). However, the lowest concentration of A23187 tested (0.05 $\mu$M), was significantly inhibitory to secretion in TE-stimulated testes, and of doubtful effect at higher concentrations (Fig. 3, dotted line).

*Calcium titers of tissues and extracts*

Although the calcium concentration of the hemolymph of *H. virescens* larvae was approximately 7 mM, $Ca^{2+}$ in whole testes was only 1–3 $\mu$M (Table 1). The calcium content of brain extracts used in these experiments was approximately 1000-times lower than that of testes, and therefore added little calcium to the incubation media (Table 1).

## DISCUSSION

The optimal calcium concentration for ecdysteroid production was approximately 1 mM for fused testis sheaths. Testes stimulated with the brain peptide, TE, synthesized ecdysteroid at approximately 0.3 mM calcium, which was significantly lower than for testes previously stimulated to synthesize ecdysteroid *in vivo*. The calcium requirements for synthesis of ecdysteroid by testes were quite different from those of the prothoracic glands of *Manduca sexta* (Smith and Gilbert, 1986; Smith *et al.*, 1987), where exogenous calcium was essential for synthesis of ecdysteroid in response to PTTH. Glands exposed to PTTH increased ecdysteroid production correspondingly as the $Ca^{2+}$ titer was increased to as much as 10 mM (Smith and Gilbert, 1986; Smith *et al.*, 1987). In contrast, ecdysteroid synthesis was inhibited at calcium concentration greater than 1.5 mM for fused testis sheaths and greater than 1 mM for testes stimulated by TE. The response of *H. virescens* testis tissue to calcium was similar to that of locust corpora allata

Table 1. $Ca^{2+}$ content of tissues and tissue extracts

| Tissue or extract | Composition of each two samples | Molarity + SEM |
|---|---|---|
| Hemolymph, *H. virescens* larvae, last stadium, stage D3–D4 | 100 μl pooled from eight male larvae | 7.0 ± 0.20 mM |
| Whole testes, *H. virescens* larvae, last stadium, stage D3–D4 | Eight testes | 2.2 ± 0.07 μM |
| Whole testes, *H. virescens* larvae, last stadium, stage B5 | One sample of four testes | 1.30 μM |
| Brain extract, *L. dispar* (in acid methanol) | 100 μl | 1.4 ± 0.39 nM (per brain equivalent) |
| Calcium-free Ringer | 100 μl | 4.6 ± 0.80 μM |

which could synthesize and release JH in calcium free media (Dale and Tobe, 1988a,b). However even more JH was synthesized in media containing 1.3 mM calcium, whereas ecdysteroid synthesis by TE-stimulated testes of *H. virescens* was inhibited in this range.

Verapamil is reputed to bind at a particular site on the calcium channel and acts to restrict the inward flow of $Ca^{2+}$ into cells (Rosenberger and Triggle, 1978; Garcia *et al.*, 1986). High titers ($10^{-3}$ M or more) of verapamil appeared to block immunodetectable ecdysteroid secretion by testes. Dale and Tobe (1988b) similarly report high titers ($10^{-3}$ M) of verapamil toxic to locust corpora allata, stopping JH production. High titers of verapamil affect the $Na^+$ and $K^+$ currents which affect calcium entry in vertebrate cardiac fibers, and may have other effects on $Na^+$ current in other vertebrate systems (Rosenberger and Triggle, 1978). It is possible that verapamil was toxic to testis sheath cells of *H. virescens* at concentrations of $10^{-2}$ to $10^{-3}$ M as well. A 10-fold decrease in verapamil concentration, however, induced an approximately six-fold increase in synthesis of immunodectable ecdysteroid by fused testis sheaths; synthesis was increased approximately three-fold at $10^{-4}$ to $10^{-11}$ M verapamil in testes stimulated by TE. In somewhat comparable fashion, locust corpora allata synthesized juvenile hormone in the absence of external $Ca^{2+}$ and at concentrations of verapamil less than $10^{-3}$ M (Dale and Tobe, 1988b). Verapamil usually inhibits calcium influx-dependent systems in vertebrates at $10^{-6}$ to $10^{-8}$ M (Rosenberger and Triggle, 1978). However, the calcium channel receptor protein is immunologically different in vertebrate and invertebrate tissue (Fosset and Ladzunski, 1987) and it may be possible that calcium channels in insect testis sheath cells have somewhat different sensitivities to these agents than those of vertebrate cells. The data presented here suggest that testis tissue from *H. virescens* secretes ecdysteroid efficiently at low external titers of $Ca^{2+}$ and under conditions where influx of calcium is inhibited.

Increasing titers of calcium ionophore A23187 correspondingly stimulated ecdysone production by *M. sexta* prothoracic glands in the presence of PTTH (Smith and Gilbert, 1986), and greatly increased the synthesis and release of juvenile hormone by the corpora allata of *L. migratoria* (Dale and Tobe, 1988a,b), indicating a dependence on calcium movement across the secretory cell membranes. One would expect, from the calcium titer and verapamil data from *H. virescens* that increased $Ca^{2+}$ traffic caused by A23187 might severely inhibit ecdysteroid secretion in both types of testis tissue as the concentration was increased. Nevertheless, A23187 had slight stimulatory effects on ecdysteroid production by fused testis sheaths, and only clearly inhibited ecdysteroid production by TE-stimulated testes at 0.05 μM. However, effects of A23187 on vertebrate steroidogenesis are variable; although A23187 increased steroidogenesis in swine ovarian cells (Veldhuis and Klase, 1982), calcium loading did not result in increased steroidogenesis in rat adrenocortical cells (Podesta *et al.*, 1980).

The response of lepidopteran testes to exogenous $Ca^{2+}$ appears somewhat like that of vertebrate Leydig cells (Dufau, 1988) or insect salivary glands (Berridge and Lipke, 1979; Berridge, 1983) which function optimally at low titers of calcium, rather than insect prothoracic glands (Smith *et al.*, 1987) which synthesize their hormonal products better at high calcium titers. Low external calcium titers regulate steps in steroidogenesis in vertebrate Leydig cells, such as interaction with the modulating neuropeptide, luteinizing hormone releasing hormone (Sullivan and Cooke, 1984), and transport of cholesterol into Leydig cell mitochondria (Dufau, 1988). Low $Ca^{2+}$ flux controls hormone-receptor attachment via GTP-induced phosphorylation of a membrane protein which complexes with luteinizing hormone and associated adenylate cyclase (Dufau, 1988). If TE-receptor coupling in lepidopteran ecdysteroidogenic cells is regulated in a comparable fashion, one would expect cells in the process of stimulation by hormones to be more sensitive to external $Ca^{2+}$ titer and $Ca^{2+}$ flux than cells in which sufficient hormone-receptor coupling had already occurred. Evidence for a comparable mechanism in the lepidopteran testis system is provided by data showing that sheaths of fused testes, activated to synthesize ecdysteroid before excision (Loeb *et al.*, 1987), functioned optimally *in vitro* at a higher calcium titer than testes stimulated by TE. Complex controls over ecdysteroidogenic mechanisms may exist as well in male insect reproductive systems.

The calcium titer (7 mM) of the hemolymph of *H. virescens* is low compared to the hemolymph of other lepidopteran species, which can be as high as 25 mM (Shaw and Stobbart, 1963). However, the amount of free calcium in the hemolymph may actually be 15–20% lower than the total calcium content due to binding by the abundant supply of proteins, amino acids and other calcium binding components (Taylor, 1986). We report a calcium titer approximately 3000-times less than that of the hemolymph in whole testes of *H. virescens*. This is not unusual, since cytosols of vertebrate cells are maintained at approximately 0.1 μM calcium against a 1000-fold blood concentration gradient. Salivary

gland cells of dipterous insects, *Chironomus* and *Calliphora*, are similarly regulated (Berridge, 1983). Multiple cellular mechanisms, including ATP-dependent cell membrane calcium exchange and modulation of calcium channels, sustain these gradients (Rasmussen, 1983; Carafoli, 1988). The interior of lepidopteran testes is protected from the environment by a blood–testis barrier (Szollozi, 1982), which may aid in maintaining a low calcium milieu for the inner layer of cells which are associated with testis ecdysteroid (Loeb, 1986). In preparation for these experiments, testes were "cleaned" of adherent tracheae, fat body, spermduct, and other tissues with sharp forceps, perhaps violating the outer cell layer or basement membrane, allowing entrance of ions. It is interesting to speculate that although the steroidogenic cells of *H. virescens* testes require low calcium media in order to synthesize ecdysteroid *in vitro*, they were protected from the contents of the hemolymph *in vivo* by a physiological barrier and thus were able to function as endocrine organs.

## SUMMARY

Testes of the lepidopteran, *Heliothis virescens*, secrete ecdysteroid optimally at low titers of $Ca^{2+}$, and increase ecdysteroid secretion when calcium channels are blocked by verapamil. This is directly in contrast to prothoracic glands of *Manduca sexta* which secrete ecdysteroid in high titers of $Ca^{2+}$ and under conditions of calcium transport mediated by ionophores. Whole testes are maintained physiologically approximately 3000-fold lower in calcium than surrounding hemolymph, implying the presence of calcium regulatory mechanisms to maintain the high gradient between the hemolymph and testis cells.

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## REFERENCES

Berridge M. J. (1983) Calcium as a second messenger. In *Endocrinology of Insects* (Edited by Downer R. and Laufer H.), pp. 615–624. Alan R. Liss, New York.

Berridge M. J. and Lipke H. (1979) Changes in calcium transport across *Calliphora* salivary glands induced by 5-hydroxytryptamine and cyclic nucleotides. *J. exp. Biol.* **78,** 137–148.

Borst D. W. and O'Connor J. D. (1974) Trace analysis of ecdysones by gas–liquid chromatography, radioimmunoassay and bioassay. *Steroids* **24,** 637–655.

Carafoli E. (1988) Membrane transport of calcium: an overview. *Meth. Enzymol.* **157,** 3–11.

Dale J. F. and Tobe S. S. (1988a) Differences in the stimulation by calcium ionophore of juvenile hormone III release from corpora allata of solitarious and gregarious *Locusta migratoria*. *Experientia* **44,** 240–242.

Dale J. F. and Tobe S. S. (1988b) The effect of a calcium ionophore, a calcium channel blocker and calcium-free medium on juvenile hormone release *in vitro* from corpora allata of *Locusta migratoria*. *J. Insect Physiol.* **34,** 451–456.

Dufau M. L. (1988) Endocrine regulation and communicating functions of the Leydig cell. *A. Rev. Physiol.* **50,** 483–508.

Fabiato A. (1988) Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Meth. Enzymol.* **157,** 378–417.

Fosset M. and Ladzunski M. (1987) Biochemical characterization of the skeletal muscle calcium channel. *Receptor Biochem.* **9,** 141–159.

Garcia M. L., King V. F., Siegl P. K. S., Rueben J. P. and Kaczorowski G. J. (1986) Characterization of diltiazem-binding sites and their interaction with dihydropyridine and aralkylamine receptors. *J. biol. Chem.* **261,** 8146–8157.

Gelman D. B., Woods C. W., Loeb M. J. and Borkovec A. B. (1989) Ecdysteroid synthesis by testes of 5th instars and pupae of the European corn borer, *Ostrinia nubilalis*. *Invert. reprod. Dev.* **15,** 177–184.

Gilbert L. I., Goodman W. and Bollenbacher W. E. (1977) Biochemistry of regulatory lipids and sterols in insects. *Int. Rev. Biochem.* **14,** 1–50.

Lange A. B., Orchard I. and Lamm W. (1987) Mode of action of proctolin on locust visceral muscle. *Archs Insect Biochem. Physiol.* **5,** 285–295.

Loeb M. J. (1986) Ecdysteroids in testis sheaths of *Heliothis virescens* larvae: an immunocytochemical study. *Archs Insect Biochem. Physiol.* **3,** 173–180.

Loeb M. J., Brandt E. P. and Birnbaum M. J. (1984) Ecdysteroid production by testes of the Tobacco Budworm, *Heliothis virescens*, from last larval instar to adult. *J. Insect Physiol.* **30,** 375–381.

Loeb M. J., Brandt E. P. and Woods C. W. (1986) Effects of exogenous ecdysteroid titer on endogenous ecdysteroid production *in vitro* by testes of the tobacco budworm, *Heliothis virescens*. *J. exp. Zool* **240,** 75–82.

Loeb M. J., Brandt E. P., Woods C. W. and Bell R. A. (1988) Secretion of ecdysteroid by sheaths of testes of the gypsy moth, *Lymantria dispar*, and its regulation by testis ecdysiotropin. *J. exp. Zool* **248,** 94–100.

Loeb M. J., Brandt E. P., Woods C. W. and Borkovec A. B. (1987) An ecdysiotropic factor from brains of *Heliothis virescens* induces testes to produce immunodetectable ecdysteroid *in vitro*. *J. exp. Zool* **243,** 275–282.

Loeb M. J and Hayes D. K. (1980) Critical periods in the regulation of the putal molt of the Tobacco Budworm, *Heliothis virescens*. *Ann. entomol. Soc. Am.* **73,** 679–682.

Loeb M. J. and Woods C. W. (1989) Metabolism of ecdysteroid in testes of the Tobacco Budworm *Heliothis virescens*. *Archs Insect Biochem. Physiol.* **10,** 83–92.

Loeb M. J., Woods C. W., Brandt E. P. and Borkovec A. B. (1982) Larval testes of the Tobacco Budworm: a new source of insect ecdysteroids. *Science* **218,** 896–898.

Maddrell S. H. P. (1980) The control of water relations in insects. In *Insect Biology in the Future* (Edited by Locke M. and Smith D. S.), pp. 179–199. Academic Press, New York.

Pressman B. C. (1976) Biological applications of ionophores. *A. Rev. Biochem.* **45,** 501–530.

Podesta E. J., Milani, A., Steffen H. and Neher R. (1980) Steroidogenic action of calcium ions in isolated adrenocortical cells. *Biochem. J.* **186,** 391–397.

Rasmussen H. (1983) Pathways of amplitude and sensitivity modulation in the $Ca^{2+}$ messenger system. Calcium and cell function. *Molec. Biol.* **4,** 2–61.

Rosenberger L. and Triggle D. J. (1978) Calcium, calcium translocation and specific calcium antagonists. In *Calcium and Drug Action* (Edited by Weiss G. B.), pp. 3–31. Plenum Press, New York.

Shaw J. and Stobbart R. H. (1963) Osmotic and ionic regulation in insects. *Adv. Insect. Physiol.* **1,** 315–399.

Shimizu T., Moribayashi A. and Agui N. (1985) *In vivo* analysis of spermiogenesis and testicular ecdysteroids in

the cabbage army worm, *Mamestra brassicae* L. *Appl. entomol. Zool.* **20,** 56–61.

Smith W., Combest W. L., Rountree D. B. and Gilbert L. I. (1987) Neuropeptide control of ecdysone biosynthesis? *Molec. Entomol.* **19,** 129–139.

Smith W. and Gilbert L. I. (1986) Cellular regulation of ecdysone synthesis by the prothoracic glands of *Manduca sexta*. *Insect Biochem.* **16,** 143–147.

Steele J. E. (1980) Hormonal modulation of carbohydrate and lipid metabolism in fat body. In *Insect Biology in the Future* (Edited by Locke M. and Smith D. S.), pp. 253–277. Academic Press, New York.

Sullivan M. H. F. and Cooke B. A. (1984) The effect of calcium ion on the potentiation of LH-stimulated cyclic AMP production by LHRH agonist ICI 118630 in rat Leydig cells. *Molec. Cell Endocrin.* **34,** 17–22.

Szollozi A. (1982) Relationship between germ and somatic cells in the testes of locusts and moths. In *Insect Ultrastructure* (Edited by King R. C. and Akai H.), pp. 32–60. Plenum Press, New York.

Taylor C. W. (1986) Calcium regulation in insects. *Adv. Insect Physiol.* **19,** 155–186.

Truman J. W. (1980) Cellular aspects of eclosion hormone action on the CNS of insects. In *Receptors for Neurotransmitters, Hormones and Pheromones in Insects* (Edited by Satelle D. B., Hall L. M. and Hildebrand J. G.), pp. 223–232. Elsevier/North Holland Biomedical Press, Amsterdam.

Veldhuis D. and Klase P. A. (1982) Mechanisms by which calcium ions regulate the steroidogenic actions of luteinizing hormone in isolated ovarian cells *in vitro*. *Endocrinology* **111,** 1–6.